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CONTRIBUTION TO THE CARBOHYDRATE CHEMISTRY OF MAPLE SAP AND SIRUP[a]

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Reports in the literature dealing with the carbohydrates of maple sap have been mainly limited to sources of the sugar, relationships of tree structures and functions to sugar formation, causes of sap flow and other factors influencing the sugar content. These indicate sap to contain only a few sugars of which sucrose is highly preponderant. Jones *(10)* reported hexoses, as represented by the reduction of Fehling's solution, in small quantities ranging from 0.003% to 0.081% with an average for 50 samples of 0.021%. Holgate *(8)* reported 0.0005 to 0.0025% invert sugar in sterile sap collected from the same tap hole as a season progressed. Bois and Nadeau *(1)* have reported the presence of cellobiose and suggest that this compound may be responsible for the small reducing power of sap.

This paper reports investigations, made as part of a study of color and flavor development during the processing of sap to sirup, which indicate that the carbohydrates consist of a relatively intricate mixture of oligosaccharides.

MATERIALS AND METHODS

Materials. The maple sap for these experiments was obtained from hard maple trees *(Acer saccharum)* in a sterile condition by means of a modification *(11)* of the technique of Holgate *(8)*. All samples of sap were checked for sterility by plating on nutrient agar. Any samples producing more than one colony per ml. were rejected. Only sap from runs collected in the 12 hours prior to processing were used. Strict sterility is necessary to prevent contamination of the sap with fermentation products.

Ion exchange methods. The fresh sterile sap was deionized by exchange resins *(12)* to obtain a neutral or carbohydrate fraction. To remove basic ions, 1700 ml. of cation resin (Dowex 50[c]) in a spherical form was employed in the [H^+] ion cycle in a glass ion-exchange column, 2 inches in diameter and 60 inches long, equipped for down flow exhaustion and regeneration and for back-washing at 50% bed expansion. A flow rate of 0.26 ml. per minute per ml. of resin was used throughout the experiments. For regeneration, 4 bed-volumes of 2N HCl were used, and the column was washed with distilled water until the effluent was free of chloride ions.

The acidic ions were removed with 1700 ml. of an amine-type anion exchange resin (Duolite A-4) in the [OH^-] ion cycle. The column dimensions and flow rate were the same as for the cation column. For regeneration, 4 bed-volumes of 1N NH_4OH were used, and the column was washed with distilled water until the effluent was acid to phenolphthalein.

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[c] Mention of commercial products does not imply endorsement by the Department of Agriculture over others of similar nature not mentioned.

Chromatographic methods. The choice of methods and the order of their use were dictated by the fact that the exceedingly large excess of sucrose over the total of all other carbohydrates made it difficult to obtain fractions of the different sugars which were completely free of sucrose. The major portion of the sucrose was removed by a preliminary resolution on the carbon column of Whistler and Durso *(14)*. Paper chromatograms were used both for isolation of fractions and for identification experiments. The solvents used were 1-propanol, ethyl acetate, water [7-1-2] *(6)*, 1-butanol, pyridine, water [3-1-1.5] *(9)* and 1-butanol, ethanol, water [10-1-2] *(16)*. Sprays employed included benzidine-citric acid in butanol *(15)* as a general reagent for all sugars, napthoresorcinol-phosphoric acid in 95% ethanol *(3)* as a ketose detecting reagent, and triphenyltetrazolium chloride *(15)* as a reducing sugar reagent. The large-scale partition method on cellulose powder, suggested by Gross and Albon *(6)* was used for the separation and study of the monosaccharides. A paper ionographic method using a laboratory constructed apparatus and conditions and procedures modified from several techniques in the literature *(4)* were used to effect resolution of several sugars appearing as chromatographic entities.

Enzyme and acid hydrolysis. An ultramicro technique for the enzymatic hydrolysis of sugars prior to papergram analysis *(13)* was employed in most cases. No buffer was necessary with the invertase[d] and invertase plus melibiase[e] enzyme preparations used at the sugar concentrations under investigation. Equal volumes of sugar solution [5-10 mg./ml.] and enzyme solution [10 mg./ml.] were employed. Mild acid hydrolyses were made using 0.05 N HCl at room temperature for one hour. Strong acid hydrolyses consisted of heating at 100°C. in 1N HCl for one hour.

PROCEDURE AND RESULTS

Experimental. Twenty gallons of sterile sap (2% solids) were freed of ions by passing the sap solution through the cation column at the stated flow rate and the effluent was passed directly into the anion column by means of a glass tubing connection. The 2 columns were washed in series with distilled water until the final effluent was free of sugars. The eluate and washings from the 2 columns were concentrated to 66.5% solids in vacuo at a temperature less than 35°C. as fast as delivered from the columns. This concentrate was designated the *neutral fraction.*

Monosaccharides. The original sterile maple sap, free of fermentation products, after concentration in vacuo, showed a slight reducing power by Schoorls alkaline copper method *(2)* which was equivalent to about 0.002% invert sugar on a sap basis after correcting for the amount of reduction due to the amount of sucrose present. The water eluate from a carbon column resolution showed a varying amount of reducing power depending upon the flow rate of an individual column. This amount of reduction, when calculated as percentage of invert in the sap, was always more than the 0.002% of reducing substance, as invert sugar, found in the original sap. This indicated hydrolysis during the carbon column resolution.

To determine the presence or absence of pentoses or hexoses, 20 g. of deionized neutral fraction was placed on a large scale cellulose column (7 cm. by 37 cm.) and resolved with 2-propanol, 1-butanol, water [7-1-2] according to Gross and Albon *(6)*. Fractions of 125 ml. each, taken by means of an automatic fraction cutter, were evaporated to dryness and taken up in 0.1 ml. of water. Papergram analysis of each successive fraction failed to detect the presence of monosaccharides. As a further check, all of these fractions (from the first to those showing sucrose) were combined, evaporated to dryness and dissolved in 0.1 ml. of water. Papergram analysis of this composite fraction also failed to show the presence of monosacharides. A mixture of equal parts of glucose and fructose when added, to the extent of 0.005% of the sucrose present (0.0001% on a sap basis), to the neutral fraction was easily detected by papergram analysis after combining all of the fractions up to sucrose from a cellulose column, evaporation to dryness and solution in 0.1 ml. of water.

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[d] Invertase, melibiase free from top fermenting yeast, Wallerstein Laboratory, gelatin vehicle.

[e] Invertase plus melibiase from fermenting yeast, Nutritional Biochemicals Corporation, gelatin vehicle.

Oligosaccharides. To each of 5 carbon-celite columns (3.4 cm. by 17 cm.), determined by analysis to have sucrose capacities varying from 2.33 g. to 2.45 g., was added 25 g. of the deionized neutral fraction (16.63 g. solids) diluted to about 20% solids with water. Five columns were used to obtain the sugars in sufficient quantities for further purification and study. Each column was eluted first with 5 liters of water, then with 4 liters of 5% ethanol. Those eluates were discarded since they contained, by papergram analysis, only mono- and disaccharides (mainly the excess of sucrose due to the overloading of the columns). The oligosaccharides were eluted completely from each of the columns with 1.5 liters of 50% ethanol. The 5 eluates were combined and concentrated in vacuo (less than 35°C.) to a small volume and freeze-dried (weight = 0.4187 g.). Papergrams indicated appreciable quantities of sucrose still to be present. A 20% water solution of this freeze-dried material was prepared and refractionated on a new carbon column by eluting successively with 1.8 liters of water, 3 liters of 5% ethanol and 2 liters of 50% ethanol. The water and 5% ethanol eluates were again discarded.

The 50% ethanol eluate was concentrated in vacuo and freeze-dried to give a residue weighing 197.7 mg. Papergram analysis indicated the presence of 5 oligosaccharide fractions, one of which was shown to be sucrose. A portion of this material (128.9) mg.) was dissolved in water and streaked on a large filter paper sheet (Whatman No. 1, 14 × 22½ inches) as a narrow band (5 mm.) and the papergram was developed with the 1-butanol, pyridine, water reagent for 24 hours. The zone positions were detected by the usual means and excised. The oligosaccharide fraction was eluted from each of the resulting paper strips with water and the extracts were dried by freeze-drying. These dried extracts were chromatographed on paper using 2 other solvents and appeared to be homogeneous.

These 5 fractions and their hydrolytic products are described in Table 1. Zone No. 1 was shown to be a trace of sucrose.

TABLE 1

Description of oligosaccharides isolated by the carbon technique and by filter paper chromatography from 83.2 g. (dry wt.) of neutral fraction

Zone No.	Rs[1]	Wt. of extracted material, mg.	% of neutral fraction	Products of complete hydrolysis with HCl[2]	Products of mild HCl hydrolysis[3] or invertase
1	1.00	8.6	0.0069	Glucose + fructose[5]	Glucose + fructose
2	0.80	17.7	0.014	Glucose + fructose	Glucose + fructose
3	0.51	20.3	0.016[4]	Glucose + galactose + fructose	Unk. oligosaccharide + fructose
4	0.20	19.3	0.015	.....	.....
5	0.00	38.8	0.031	Glucose + galactose + fructose	.....

[1] Mobility compared to sucrose in 1-butanol-pyridine-water.
[2] 1 N HCl at 100°C. for 1 hr.
[3] 0.05 N HCl at 25°C. for 1 hr.
[4] Equivalent to less than 0.0005% of the original sap.
[5] Monosaccharides identified by comparison with known compounds.

The oligosaccharide from Zone No. 3 was hydrolyzed with invertase, streaked on filter paper and chromatographed with 1-butanol, pyridine, water. A new oligosaccharide and fructose were resolved. This oligosaccharide was eluted from the paper. Chromatography in two other solvents gave no further separation and so indicated homogeneity. Preliminary paper ionographic experiments *(4)* on the original trisaccharide [0.05 M. borate, pH 9.2, 7 v./cm., 3.6-4.2 m.a.] indicated two major and possibly two very minor components. Analysis by the same method of the oligosaccharide fraction derived from the original by invertase indicated the presence of two compounds, one of which had the same mobility as melibiose, the only disaccharide run as a known.

The results of the action of the different hydrolyzing reagents on this derived oligosaccharide fraction and the unhydrolyzed trisaccharide are given in Figure 1.

A sample of the original extract from Zone No. 3, weighing approximately 200 μg., was hydrolyzed with invertase and chromatographed on paper. The disaccharide spot and the fructose spot were excised and eluted with water. The disaccharide sample

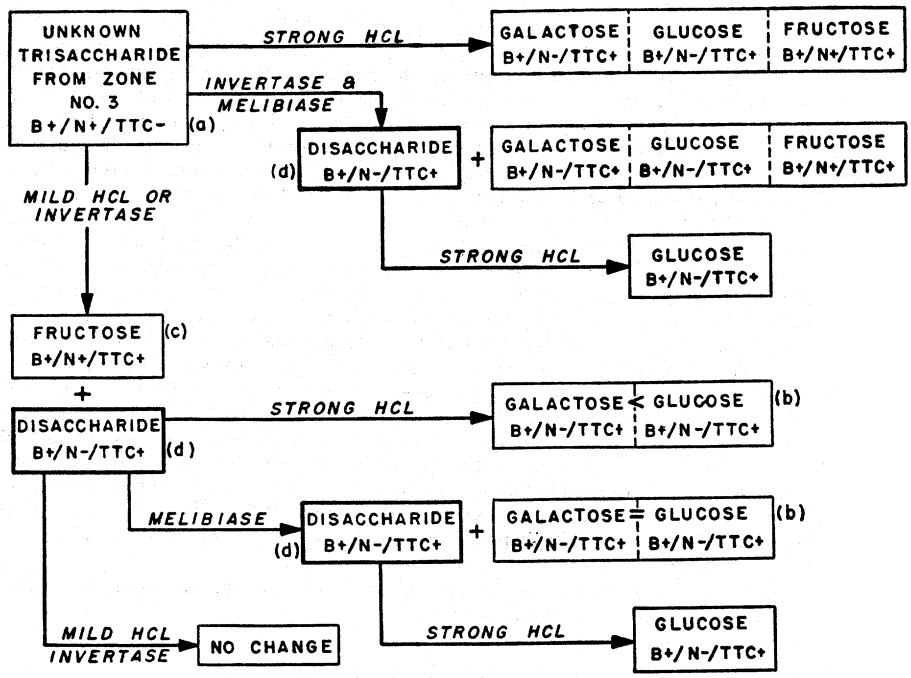

Figure 1. The enzymic and acid hydrolytic products of the trisaccharides isolated from zone No. 3.

(a) Indicator reagents: B = Benzidine-citric acid; N = Naphthoresorcinolphosphoric acid; TTC = Triphenyltetrazolium chloride.

(b) Semi-quantitative measurements of glucose and galactose made by visual comparison of the benzidine developed spots with standards of equal parts of glucose and galactose run simultaneously on the chromatogram.

(c) Estimated by micro reduction to be in a ratio of one mole of fructose to one mole of disaccharide.

(d) Each system of blocks indicates spots observed on a papergram as a result of hydrolysis of the starting material in question. Those components not isolated are separated by dotted lines. The heavily outlined blocks indicate this material was isolated from the hydrolytic products by paper chromatography for the purpose of further study.

was hydrolyzed by refluxing at 100°C. in 1N HCl for one hour and neutralized with NaOH. Shaffer and Somogyi microdetermination *(2)* of the sugars showed 70 μg. of fructose and 135 μg. of sugars as glucose from the disaccharide indicating one mole of fructose per mole of disaccharide or one mole of fructose per mole of original trisaccharide.

Rechromatography of the original extract from Zone No. 3 on carbon indicated the unhydrolyzed compounds in the mixture to be trisaccharides since they were eluted with 15% ethanol. The oligosaccharide fraction, produced by the action of invertase on the original extract from Zone No. 3 was eluted from another carbon column with 5% ethanol, indicating that only disaccharides were present.

DISCUSSION

The formation of monosaccharides during resolution of the neutral fraction of sterile sap by use of carbon columns has not been explained. Slight acidity of the carbon combined with the enormous surface furnished by the carbon particles may be the explanation but for the fact that solu-

tions of pure sucrose passed through the same column did not yield any increased amount of invert sugar over that present in the sucrose before chromatographing. A further possibility is the hydrolysis of some oligosaccharide which is more labile than sucrose. As identification of the several sugars progresses, their stability on the carbon column will be studied.

When extremely mild conditions were employed (partition chromatography on cellulose powder) no monosaccharides could be detected in the sterile maple sap (0.0001% of invert sugar added to the sap could be easily detected). Sap collected under non-sterile conditions but processed within 36 hours showed a detectable amount of glucose and fructose. As microbial contamination increased, proportionally more glucose and fructose were detected confirming the results of Holgate *(8)* and Hayward *(7)*.

The chromatographic and hydrolytic data indicate that sterile sap contains, in addition to sucrose, at least 5 other oligosaccharides. The experience with the original extract from Zone No. 3, which was apparently homogeneous by chromatographic criteria, but which was shown to be a mixture of two trisaccharides by hydrolytic and paper ionographic techniques, lends doubt to the homogeneity of the 3 other fractions. Therefore, there may be more than 5 oligosaccharides in addition to sucrose. Results similar to these were reported by Gross *(5)* in his study of the paper electrophoresis of oligosaccharides synthesized from sucrose by yeast invertase.

Examination of the results reported in Figure 1 for the sugars isolated from Zone No. 3 shows the following:

a. The trisaccharides produce galactose, glucose and fructose upon strong acid hydrolysis.

b. Invertase plus melibiase hydrolysis produces galactose, glucose and fructose plus a disaccharide which yields only glucose upon hydrolysis with strong acid; therefore, one of the trisaccharides must have two adjoining glucose units.

c. Mild acid or invertase hydrolysis causes the formation of a disaccharide mixture and fructose which by analysis were shown to be in a 1:1 ratio. Therefore, each trisaccharide unit must contain a fructose unit. Hydrolysis with invertase indicates that each trisaccharide must have a sucrose unit with a terminal fructose.

d. Melibiase hydrolyzes one part of the disaccharide mixture resulting from the mild acid hydrolysis to yield galactose and glucose in equal proportions but does not hydrolyze the other disaccharide. Since melibiase is specific for an α-galactoside linkage, one trisaccharide must consist of melibiose plus fructose and since it contains a sucrose linkage it can be concluded that it is probably raffinose or a closely related compound. The disaccharide remaining after melibiase hydrolysis yields only glucose when hydrolyzed with strong acid. Therefore, the second trisaccharide must consist of a diglucose disaccharide and fructose and since it contains a sucrose linkage it is a glucosyl sucrose.

R_s data on the diglucose disaccharide [0.41 in 1-propanol, ethyl acetate, water], when compared to maltose [0.69] and cellobiose [0.61], eliminated it as being one of these. Further work to characterize the linkages for final identification of this sugar is in progress.

The characterization of the materials isolated from Zones numbered 2, 4 and 5 will be the subject of future papers.

SUMMARY

Maple sap, if collected under sterile conditions, contains less than 0.0001% by weight of monosaccharides. It has been demonstrated to contain at least five oligosaccharides in addition to sucrose. One fraction isolated by paper chromatography and apparently homogeneous by chromatographic standards was shown to be a mixture of two trisaccharides which lends credence to the possibility that other fractions may be mixtures. One of the two trisaccharides of the mixed fraction has been established to be raffinose or a closely related compound and the second to be a glucosyl sucrose.

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